

Jörgen Malmquist

**LEUKOCYTIC PROTEINS
IN SERUM AND URINE:
PATTERNS IN
LEUKEMIA AND POLYCYTHEMIA**

Malmö 1972



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LEUKOCYTIC PROTEINS IN SERUM AND URINE:

PATTERNS IN LEUKEMIA AND POLYCYTHEMIA

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Jörgen Malmquist

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From the Department of Medicine
(Head: Professor Jan Waldenström)
University of Lund, Malmö General Hospital
Malmö, Sweden

LEUKOCYTIC PROTEINS IN SERUM AND URINE:
PATTERNS IN LEUKEMIA AND POLYCYTHEMIA

by

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This thesis is a summary of the following publications:

- I B.G. Johansson & J. Malmquist: Quantitative immuno-chemical determination of lysozyme (muramidase) in serum and urine. Scand. J. clin. Lab. Invest. 27: 255, 1971.
- II J. Malmquist & B.G. Johansson: Interaction of lactoferrin with agar gels and with Trypan Blue. Biochim. Biophys. Acta 236: 38, 1971.
- III J. Malmquist: Serum and urinary lysozyme in leukaemia and polycythaemia. To appear in Scand. J. Haemat.
- IV J. Malmquist: Serum lactoferrin in leukaemia and polycythaemia. To appear in Scand. J. Haemat.
- V J. Malmquist: Serum myeloperoxidase in leukaemia and polycythaemia. To appear in Scand. J. Haemat.

These papers will be referred to by the above Roman numerals.

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INTRODUCTION

NORMAL LEUKOCYTE TURNOVER

Knowledge on rates and mechanisms of white blood cell turnover is of obvious importance in clinical hematology. For the normal neutrophilic granulocyte series a considerable amount of data on pool sizes and turnover rates is available largely through the studies by Wintrobe and associates with the ^{32}P -DFP labeling technique (Cartwright & al., 1964). No quantitative data exist for eosinophilic and basophilic granulocytes. For the monocyte series turnover data have become available more recently (Fliedner & al., 1969). The lymphocyte series has been studied in great detail, but because of the heterogeneity of this population with respect to survival times, and because of the extensive recirculation of these cells between various anatomic pools, lymphocyte turnover cannot be described in simple quantitative terms.

For all leukocyte groups there is a lack of adequate information about the mechanisms of cell elimination (Boggs, 1967). It is generally agreed that granulocytes as well as monocytes migrate unidirectionally from the bone marrow to the blood and from there to the interstitial tissue space and to body cavities. Their subsequent fate differs considerably. The monocyte is now believed to transform to tissue phagocytes, and to be the major, if not sole, source of such cells (Volkman, 1970). These morphologically transformed monocytes have a considerable tissue life span, although quantitative data have not been produced.

In contrast, the fate of the granulocyte after it leaves the blood is rather enigmatic. It is generally agreed that only a small minority of granulocytes die intravascularly (Fliedner & al., 1964). The majority traverse capillary walls to fulfil their phagocytic functions. However, it has not been possible to measure their tissue life span, which seems to be quite brief; indeed, the existence of a significant normal tissue pool of granulocytes cannot be demonstrated (Boggs & al., 1964).

A calculation using accepted granulocyte turnover data indicates that a packed cell volume of at least 50 ml is disposed of daily. As remarked by Hirsch (1965) microscopic examination of normal tissues shows no signs of a leukocyte graveyard. It is known (reviewed by

Perry & al., 1968; Perry, 1971) that large numbers of granulocytes traverse pulmonary alveolar walls and intestinal mucosa to end up in bronchial secretions and intestinal contents, respectively. Additional cells enter the upper air passages, the oral mucosa, and the saliva. It is evident that, through swallowing, the majority of granulocytes from the sources mentioned will be added to those that have entered the intestinal lumen directly. Small numbers of cells (certainly less than one per cent of the total number disappearing) are disposed of in urine, through the female genital tract, and together with desquamated epidermal cells. It is the opinion of many hematologists (e.g., Hirsch, 1965) that the vast majority of granulocytes disappear by thus leaving the body, predominantly through the intestine ("external" destruction). Others (Wintrobe, 1967a) feel that disintegration and dissolution within various organs ("internal" destruction) is more likely to be the quantitatively predominant process.

Elimination through the intestine seems to account for most of the granulocyte turnover in the rat (Teir & al., 1963). In man a study of stool leukocyte content has been reported (Pitzurra & Frascarelli, 1943) but the value of these data is limited for the obvious reason that many of the cells entering the intestine can be expected to be destroyed in the intestinal lumen.

Similar considerations regarding ultimate disposal are valid for lymphocytes; again, emigration through the intestinal mucosa is believed to be a significant mechanism (Ambrus & Ambrus, 1959).

LEUKOCYTE TURNOVER IN LEUKEMIAS AND ALLIED DISORDERS

Attempts at the application of kinetic concepts in these conditions are clearly desirable, for theoretical as well as practical reasons. Present knowledge on cell pool sizes and turnover rates in leukemias derive from studies with radioisotope labeling techniques. The data thus obtained, as well as the difficulties encountered in their interpretation, have been reviewed by Craddock (1960), by Perry & al. (1968), and by Clarkson (1969). One of the principal outcomes of these studies is a reevaluation of growth mechanisms. The previous assumption that

the basic mechanism of leukemia was the excessively rapid proliferation of immature hematopoietic cells has been replaced by the now widely held opinion that the proliferation rate, on a per cell basis, is most often similar to, or lower than, the rate in normal leukocyte precursor populations. The reason for the accumulation of leukemic cells in blood and tissues must then be sought in an increased survival time, perhaps intimately related to the maturation failure. Similar considerations have been applied to the growth of solid tumors (Post & Hoffman, 1969).

Although, then, leukemic cells are probably seldom, if ever, characterized by a higher than normal proliferative activity, this does not mean that the cell production rate is not high. Whereas the average generation time within the dividing leukemic cell population is normal or prolonged, the population is most often so large that the net effect is an enhanced cell production rate.

In untreated or relapsed leukemia the cell population is, as a rule, expanding, but it can generally be surmised that an increased cell production signifies an increased cell destruction as well.

The only consistent exception to the above concepts is chronic lymphocytic leukemia, where the pathogenesis seems to involve only a prolonged cell survival, with no increase in cell production (or destruction) rate above normal (reviewed by Begemann, 1970).

The practical clinical interest in kinetic evaluation of leukemias stems largely from the desirability of monitoring the effects of cytotoxic therapy. Although radioisotope labeling techniques are in use in several clinical centers, they have the disadvantages of being quite laborious and of presenting considerable difficulties in interpretation, above all in the acute leukemias (e.g., Galbraith, 1970). A case can therefore be made for additional attempts to supplement classical hematological parameters with measurements that may give some indication, however approximate, of the magnitude of cell turnover rate. The most useful type of measurement would be one that not only provided some information related to kinetics, but also, in the individual case, could supplement morphological evidence on the nature of the cells (cell line and level of maturity) involved in the leukemic process.

THE MEASUREMENT OF LEUKOCYTE CONSTITUENTS IN BODY FLUIDS

Clinical evaluation of erythrocyte turnover is greatly aided by the measurement of free hemoglobin and its conversion products. In contrast, it has been felt that the leukocyte "has no specific catabolic products that could be used to monitor its rate of destruction" (Perry & al., 1968).

The serum lactic dehydrogenase level is often elevated in leukemias, but the correlation between enzyme concentration and disease activity is often poor, and it cannot be established with certainty that the leukemic cells are the enzyme source (Magill & al., 1959).

Uric acid production is increased in many cases of leukemia (Sandberg & al., 1956; Krakoff & Balis, 1964), and can be expected to mirror the rate of cell destruction to some extent. One disadvantage of this parameter is its non-specificity, cellular necrosis in any tissue causing some increase in uric acid production. In addition, variations in the extent of reutilization of nucleic acid fragments and in the rate of uric acid elimination (renal excretion and intestinal uricolysis) combine to make interpretations difficult.

Although lysozyme (muramidase) has been known as a component of white blood cells since its discovery by Fleming, no attempts were made to use serum lysozyme as a turnover indicator until 1959, when Inai & al. published the results of lysozyme assays in blood disorders. The principal result reported was the clearly elevated serum lysozyme concentration in chronic myelocytic leukemia. Subsequently, experiences with lysozyme determinations in blood disorders were reported by Finch & al. (1964) and by Jollès & al. (1964). Osserman & Lawlor (1966) were the first to examine urinary lysozyme. They made the important finding that large amounts, up to 2 - 3 grams daily, of the enzyme were excreted in cases of monocytic and myelomonocytic leukemia. Subsequently, a large number of reports on lysozyme studies in leukemias and related diseases have appeared.

The data available on lysozyme form a basis for some general considerations on the clinical usefulness of attempts to measure leukocytic "marker" proteins in serum and/or urine. Such studies may be expected to provide some information on one or more of the following points:

- 1) Cell pool sizes: marrow pool, blood pool, tissue pools, or combinations of these.
- 2) Rate of cell destruction.
- 3) The nature of the cells predominantly involved in a leukemic process.

Item **3**) concerns cytological differential diagnosis of leukemias and is also related to the degree of maturation of the cell species involved. Items **1**) and **2**), which are clearly interrelated, concern disease "severity" and the effects thereon of therapeutic intervention.

Apart from the obvious requirement that the proteins selected as "markers" be sufficiently leukocyte-specific, several other considerations are relevant for the interpretation of this kind of data. Cellular proteins may be released only at the ultimate destruction of the cell, or may leak out from the cell already during cell production and maturation in bone marrow or elsewhere, or during the intravascular or extravascular life span of the cell.

Release restricted to cell destruction will tend to provide data related to turnover rate. However, the sites of cell destruction will be of importance. As discussed above for normal granulocytes, an unknown proportion of the cell mass disappearing daily is actually exteriorized, above all to the intestinal tract, before disintegrating. Cell proteins thus released cannot contribute to serum and urine concentrations. (This restriction does not necessarily apply to low molecular weight non-protein cell constituents. For example, part of the increased uric acid production observed in some leukemias may originate from leukocytic nucleic acid fragments that have been reabsorbed from the intestine.)

Even though it can safely be assumed that leukocyte destruction does

to some extent take place in various tissues, only a small proportion of cell constituents thus released may ultimately reach the blood circulation. Tissue phagocytes that ingest effete leukocytes or fragments of these may digest much of the leukocytic proteins. Such protein molecules that are released intact into interstitial fluid cannot be expected to reach blood plasma by direct diffusion to any significant extent. Rather, drainage through the lymphatic system must be expected (Dumont & Weissman, 1964; Smith & al., 1970). It is certainly conceivable that losses of material occur in this process, i.e. before the point of influx of lymph into the blood circulation. Bone marrow and spleen are exceptions in this regard, being virtually devoid of a lymphatic drainage system and having instead anatomic conditions favoring access directly to the blood circulation. In conclusion, the plasma concentrations of proteins of presumed leukocytic origin can be expected to reflect only that proportion, possibly minute, of cells that are destroyed "internally", and perhaps only those cells disintegrating at particular anatomic sites, such as bone marrow and spleen.

If intravascular release from viable cells is the predominant process the serum concentrations of cell constituents will obviously not give information about cell turnover rate, but will reflect the size of the intravascular cell pool. This has been shown to be true for the vitamin B₁₂ binding protein transcobalamin I (Chikkappa & al., 1971). In addition, material released from viable cells in extravascular pools may reach the blood circulation and may thus contribute to serum concentrations.

If knowledge on the sites of leukocytes destruction ("internal" vs. "external") is deficient for the normal state, it is practically non-existent for the leukemic state. Even within a single diagnostic group considerable variations in the cell disposal pattern may possibly exist between individuals. In addition, assessment of data on released leukocytic proteins is hampered by uncertainty regarding the per cell content of such proteins. Considerable deviations from the composition of normal cells must be expected. As the most obvious property of leukemic cells is the more or less pronounced maturation failure, a reduced capacity for the production of specialized constituents might seem the most probable event. However, the opposite, i.e., a disturbance causing increased synthesis, may also be expected to occur. Thus, the disintegration of an immature leukemic cell may cause the release

of smaller than normal, normal, or larger than normal amounts of "marker" proteins.

LEUKOCYTE PROTEINS OF POSSIBLE "MARKER" STATUS

Of the different classes of leukocytes, lymphocytes and their precursors are not known to contain any quantitatively important components that can unequivocally be identified as belonging to that cell line. For this reason the lymphocytic cell series has been omitted from the following discussion.

Of proteins more or less specifically present in granulocytes and monocytes, lysozyme must be considered the prime example, in view of the wealth of data available. The amount of lysozyme per cell seems to be of the same order of magnitude in normal neutrophilic granulocytes as in normal monocytes (Briggs & al., 1966; Asamer & al., 1969; Senn & al., 1970; Syrén & Raeste, 1971). In rabbit heterophilic granulocytes it is present in both of the two major classes of cytoplasmic granules recognized in this cell, the primary and the secondary granules (Baggiolini & al., 1969, 1970a). Lysozyme is also found in tissue macrophages as well as in various exocrine glands and their secretions (reviewed by Osserman & Lawlor, 1966). The highest tissue concentration of lysozyme is found in cartilage, where its localization is predominantly extracellular (Eisenstein & Kuettner, 1970).

Lactoferrin, an iron-binding protein originally found in milk, from where it was isolated by Johansson (1960) and by Montreuil & al. (1960), has been shown recently to be present in, and synthesized by, neutrophilic granulocytes and their precursors (Masson & al., 1969). Granule fractionation studies with rabbit heterophilic granulocytes established that lactoferrin was localized in the secondary granules (Baggiolini & al., 1970b). In addition to the mammary gland, lactoferrin has been found in various other exocrine glands and in some other tissues, including kidney; in some of these sites a local synthesis has been demonstrated (Masson, 1970).

Myeloperoxidase is the enzyme responsible for the well known peroxidase staining of neutrophilic granulocytes and monocytes. Although eosinophilic granulocytes display the most marked peroxidase activity,

the eosinophil enzyme has been shown to differ by cytochemical criteria from neutrophil peroxidase (Rytömaa & Teir, 1961; Undritz, 1966; Yam & al., 1971). Proof that the eosinophil and neutrophil enzymes are not identical was provided by studies on individuals with hereditary myeloperoxidase deficiency. In this condition peroxidase activity is absent from neutrophils as well as from monocytes, but retained in eosinophils (Undritz, 1966; Salmon & al., 1970). Furthermore, a fluorescent antiserum against myeloperoxidase was found to react with normal neutrophils and monocytes, but not with eosinophils (Salmon & al., 1970). Accordingly, by genetic, cytochemical, and immunological criteria the neutrophil and eosinophil peroxidase are different entities, whereas the neutrophil enzyme is indistinguishable from that of monocytes.

Human myeloperoxidase was purified by Agner (1941) and was found to be a green-colored heme enzyme. Studies on human myeloperoxidase have more recently been reported by Schultz and associates (Schultz & Kaminker, 1962; Schultz & Shmukler, 1964; Schultz & al., 1965). It makes up a surprisingly large part of the contents of the neutrophilic granulocyte, more than 5% of the dry weight according to Schultz & Kaminker (1962). In contrast to lysozyme and lactoferrin, myeloperoxidase seems to be strictly leukocyte-specific; it has not been demonstrated in any other tissues (Schultz & Berger, 1970). Granule fractionation studies with rabbit heterophilic granulocytes indicate that myeloperoxidase is localized in the primary granules (Baggiolini & al., 1969, 1970a). Electron microscopic cytochemical studies with human leukocytes (Dunn & al., 1968) likewise suggest the localization of myeloperoxidase in the primary granules of the neutrophil, and in the azurophilic granules of the monocyte (the only kind of granule so far demonstrated in monocytes).

Although the functions of these three leukocytic proteins are not directly relevant to the present study, it may be mentioned that lysozyme and myeloperoxidase, and possibly lactoferrin, are involved in the microbicidal activity of leukocytes (Osserman & Lawlor, 1966; Klebanoff, 1970; Masson & al., 1969).

PRESENT INVESTIGATIONS

The studies to be reported had the following aims:

- 1) To purify human lysozyme, to devise an immunochemical method for its quantitation in serum and urine, and to compare the results thus obtained in leukemias and allied disorders with published results obtained by bacteriolytic methods for lysozyme assay.
- 2) To purify human lactoferrin and myeloperoxidase and to attempt the measurement of these proteins in serum by immunochemical methods. Furthermore, to ascertain whether these proteins, like lysozyme, show deviations from normal in blood diseases.

The desirability of the application of cell kinetic concepts in clinical hematology has been pointed out. As a supplement to the indispensable morphological studies and blood and bone marrow cell enumerations, attempts to estimate cell mass sizes and cell production/destruction rates can reasonably be expected to assist in the clinical evaluation of the activity of a leukemic process, including the changes therein brought about by therapy. It is hoped that the kind of data to be reported here will ultimately turn out to bear some relation to leukemic cell pool sizes and turnover rates. However, as facilities (radioisotope-labeling methods) for obtaining kinetic data were not available in the present studies, the results to be presented cannot be discussed in terms of true cellular kinetics. Rather, the studies were exploratory, aiming at a description of leukocyte marker protein data in relation to diagnostic categories and to conventional hematological parameters.

THE PURIFICATION AND MEASUREMENT OF LYSOZYME (I)

Before the discovery of lysozymuria in leukemias, human lysozyme was purified from various secretions: milk, tears, and saliva (reviewed by Jollès, 1969). The presence of large amounts of lysozyme in urines of some patients with monocytic and myelomonocytic leukemia makes such urine samples a convenient starting material for preparative procedures. Osserman & Lawlor (1966) purified human urinary lysozyme by bentonite

absorption, employing the procedure used by Alderton & al. (1945) in the purification of hen egg white lysozyme.

In the present work (I) a similar batch absorption method was used, but the cation exchanger carboxymethyl-(CM-)Sephadex was found to be more convenient than bentonite. Since lysozyme is a strongly basic protein it is firmly bound to the cation exchanger. It can subsequently be eluted by high ionic strength buffer. The procedure sacrifices yield for purity. No contaminants have been detectable in the lysozyme preparations obtained. The rather low yield (about 30%) is of no practical concern, as suitably selected urine samples contain up to several grams of lysozyme per liter.

The lysozyme obtained was used for the production of rabbit antisera and as standard in the immunochemical lysozyme determination, which was performed according to Laurell (1966) by electrophoresis into antibody-containing agarose gel.

The normal serum lysozyme concentration was found to range between 1 and 5 mg/l, whereas in normal urine the lysozyme concentration was invariably below 1 mg/l. A comparison of the present method with a bacteriolytic assay with dried Micrococcus lysodeikticus cells, using the same preparation of human lysozyme as standard, showed a good concordance between the methods.

The classical bacteriolytic (turbidimetric) lysozyme assay has the advantage of simplicity, requiring only commercially available M. lysodeikticus cells and some kind of photometric equipment. A variant of this assay is the "lyso-plate" method of Osserman & Lawlor (1966), which is somewhat analogous to radial immunodiffusion. The samples are placed in wells in an agar gel containing M. lysodeikticus. The lysozyme diffuses out in the gel and its activity becomes evident as a zone of clearing in the otherwise turbid bacterium-containing gel. The size of the cleared halo is related to the lysozyme content of the sample.

Bacteriolytic lysozyme assays are not specific enzyme assays, since the hydrolytic process (the cleavage of N-acetylmuramic acid - N-acetylglucosamine glycosidic bonds) is not measured as such; the disruption of bacteria observed is a secondary feature. The complexity of the relationship is apparent from several studies, e.g., that of Gorin & al. (1971). These considerations do not, per se, diminish the practical usefulness of bacteriolytic assay methods. On the other hand, such methods naturally require that the lysozyme is present in the samples in active condition. Since the enzyme seems to be quite stable, this is not an impor-

tant objection. Likewise, the occurrence of lysozyme-inhibitory substances in the samples has not been noted to be a problem in studies on bacteriolytic lysozyme assays. However, even if enzyme inhibition in a strict sense may not occur, such assays are still open to the objection that the phenomenon measured, the disruption of bacterial cells, may be influenced by sample components other than lysozyme. Several published observations seem compatible with this assumption (discussion in paper I). In a recent report Harrison & Swingler (1971) have described the disturbing influence of serum macromolecules on the lysis of M. lysodeikticus.

Immunochemical assay, on the other hand, require that the lysozyme is not severely denatured or otherwise changed so as to be immunologically unrecognizable. Also, the method rests on the assumption that there is no molecular variations among human lysozymes. It is generally agreed that lysozymes of various origins in normal man are identical (Osserman & Lawlor, 1966). For lysozyme originating in leukemic leukocytes, there is certainly a possibility for molecular deviations from normal. If (as in the present studies) the lysozyme used for antiserum production is obtained from a single leukemia patient, the antibodies might have a different reactivity with normal lysozyme and with other leukemia lysozymes. Although Mouton & Jollès (1969) claim to have detected heterogeneity in urinary lysozyme from a case of myeloblastic leukemia, a deviation from normal in amino acid composition was not found. No other reports on molecular variations in lysozyme have appeared. Accordingly, it is justifiable to disregard this objection at present.

The immunochemical lysozyme assay is slightly more laborious to perform than the turbidimetric assays. The latter can be adjusted to provide a somewhat higher sensitivity than the immunochemical assay, making possible, for example, measurements in part of the subnormal serum lysozyme range, where the immunochemical method is unreliable. However, the clinical importance of quantitation in these rarely encountered samples is probably small, although Wiernik & Serpick (1969) claim that variations between subnormal and normal serum lysozyme values are of some significance in the treatment of lymphoblastic leukemia.

A novel form of immunoassay for lysozyme was reported recently by Maron & Bonavida (1971). This method is quite sensitive, measuring down to about 10 µg/l, but rather complicated, employing bacteriophage-lysozyme conjugates.

In immunochemical methods the lysozyme standard is, of necessity, human lysozyme. In bacteriolytic methods, any kind of lysozyme can be used as a reference for comparison with the activity of the samples. In a large number of publications the commercially available hen egg white enzyme has been employed. Because the specific activity (activity per unit weight) of human lysozyme is 3 - 4 times higher than that of hen egg white lysozyme, assays with the latter enzyme as standard will produce concentration values that are three- to fourfold higher than the true concentration, if a correction is not applied. This fact, which has frequently been overlooked in the literature, has to be kept in mind when comparing results reported by different authors. With the lyso-plate method, the specific activity ratio human lysozyme / hen egg white lysozyme is, for obscure reasons, between 8 and 12 (Osserman & Lawlor, 1966). A systematic comparison of results obtained by the lyso-plate method with those by ordinary turbidimetry has not been reported.

LACTOFERRIN: PURIFICATION AND PHYSICO-CHEMICAL OBSERVATIONS (II)

Lactoferrin was originally believed to be present only in milk and other secretions. Its significance in the present context stems from the observation by Masson & al. (1969) of its presence in the neutrophilic granulocyte series.

In order to establish an immunoassay for lactoferrin, the protein was purified from human milk by a procedure slightly modified from that described by Johansson (1969). The method was similar to that used for urinary lysozyme, in that batch absorption to CM-Sephadex was used as the initial step. The cation exchanger with the bound proteins was washed with water and with a Tris buffer. After packing into a chromatographic column the lactoferrin could be eluted by raising the ionic strength of the buffer. Part of the milk lysozyme was obtained in the same effluent. Because of the difference in molecular size between lactoferrin (MW 76 000; Querinjean & al., 1971) and lysozyme (MW 14 600; Jollès & Jollès, 1969) the proteins could easily be separated by gel filtration on Sephadex G-75. The lactoferrin thus obtained was free of detectable contaminants; with some of the batches

produced crystallization occurred. Potent antisera were easily obtained in rabbits.

In the course of attempts to devise a sensitive immunochemical determination method a tendency of the protein to interact with a number of non-protein compounds was noted. In his detailed studies on lactoferrin Masson (1970) observed an anomalous migration in electrophoretic media, including agarose. The phenomenon was again observed in agarose electrophoresis in the present studies, and was tentatively ascribed to a binding to residual sulfated components in the agarose, a possibility already suggested by Masson (1970). In further electrophoretic studies, binding of lactoferrin to a number of polyanionic macromolecules was observed, e.g. nucleic acid (cf. Loisillier & al., 1968), heparin, and chondroitin sulfate. Although a mechanism of electrostatic attraction would seem likely, this explanation is somewhat imperfect, as the lactoferrin molecule would not be expected to have any significant predominance of positively charged groups in the pH range around neutrality. Neither physicochemical data (Masson, 1970) nor amino acid composition (Querinjean & al., 1971) places lactoferrin among "basic" proteins. The binding phenomena (including the rather firm attachment of lactoferrin to cation exchangers) may possibly be explained by markedly uneven charge distribution, i.e., a regional accumulation of positively charged groups in clusters. Regardless of the mechanism the biological significance, if any, of these interaction phenomena remains unknown.

Furthermore, the electrophoretic behavior of lactoferrin was found to be influenced by a number of dye compounds, most notably by Trypan Blue. Data obtained by electrophoresis, gel filtration, spectrophotometry, and ultracentrifugation indicated that a dye-protein complex of a constant composition was formed, and that the complex contained more than one, most probably two or three, lactoferrin molecules. The voluminous literature on Trypan Blue, one of the oldest vital stains, will not be reviewed here. In addition to its staining properties the dye has remarkable biological effects, its administration to experimental animals producing "reticulo-endothelial blockade" (Beeson, 1947), teratogenesis (reviewed by Lloyd & Beck, 1969), and tumors of the lymphoreticular cell system (Gillman & Gillman, 1949). However, any speculations on a possible relationship between the present in vitro observations and in vivo effects would be premature.

The experiments described were to some extent related to the practical problem of measuring lactoferrin in body fluids, as they pro-

vided some insight in the behavior of lactoferrin in gel diffusion and immunoprecipitation. In single radial immunodiffusion the sensitivity obtained was rather low, the detection limit being about 10 mg/l, insufficient for serum lactoferrin quantitation. The low sensitivity may be related to the affinity of lactoferrin for gel components, which can be expected to cause slow diffusion as well as blockage of combining sites. Alternatively, it may be a feature of the antigen-antibody system as such. Furthermore, the Laurell (1966) technique of electrophoresis into antibody-containing gel was found to be applicable to purified lactoferrin, and to provide a somewhat higher sensitivity than single radial immunodiffusion. However, when the procedure was attempted with lactoferrin added to sera, only indistinct precipitation lines, not coalescing to a peak, were obtained. This disturbance, not observed previously in the use of this method, was taken to be another manifestation of unusual molecular properties of lactoferrin. Accordingly, this procedure had to be abandoned. As described below (IV), the technique of single radial immunodiffusion was found to be useful, if its sensitivity was increased by the use of isotope-labeled antiserum and radioautography, as described by Rowe (1969).

LYSOZYME IN BLOOD DISORDERS (III)

The immunochemical method for lysozyme determination (I) was applied to serum and urine samples from 51 patients with leukemias and polycythemia vera. Serum lysozyme was normal or subnormal in all patients with chronic lymphocytic leukemia. In acute (non-monocytic) leukemia lysozyme values varied between normal and considerably elevated (range 2 - 60 mg/l). Serum lysozyme was increased in all patients with monocytic leukemia (33 - 130 mg/l), chronic myelocytic leukemia (6.5 - 38 mg/l), and polycythaemia vera (6 - 23 mg/l). In the two last-mentioned groups many values were close to the normal range. The distribution is shown in Fig. 1 A. The overlap between acute leukemias and monocytic leukemia is evident.

Lysozymuria was present in 9 patients. One patient with monocytic leukemia excreted between 3000 and 5500 mg of lysozyme daily and

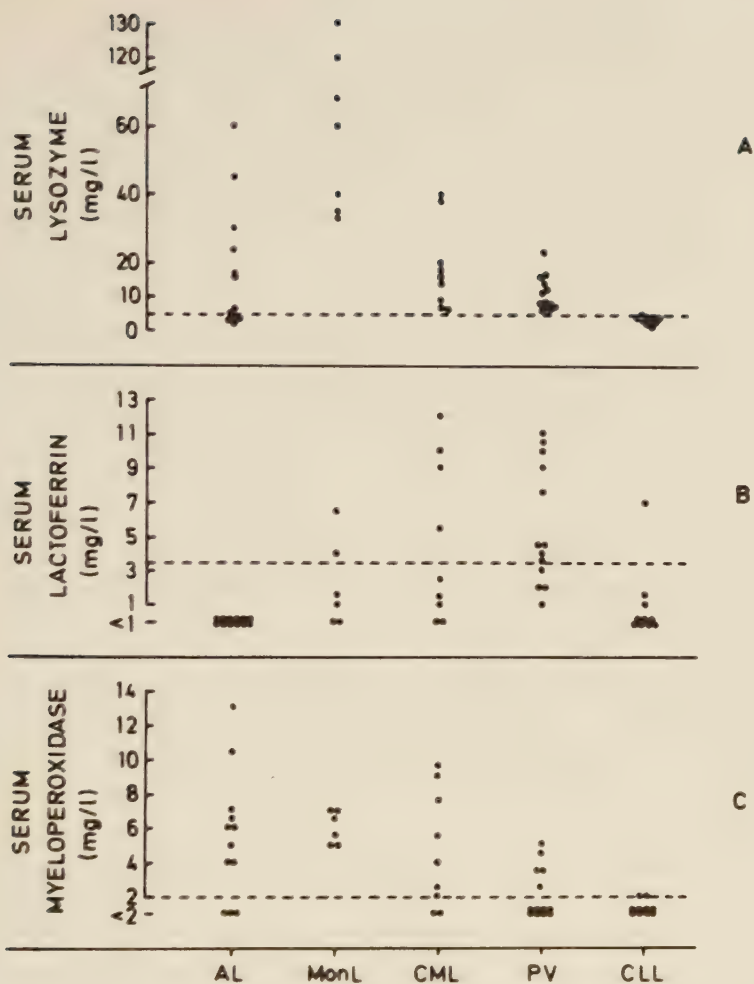


Figure 1. Serum concentrations of leukocytic proteins in blood disorders. A: lysozyme. B: lactoferrin. C: myeloperoxidase. The dashed lines indicate the upper limits of normal concentrations. AL = acute leukemia. MonL = monocytic leukemia. CML = chronic myelocytic leukemia. PV = polycythemia vera. CLL = chronic lymphocytic leukemia.

displayed a "lysozyme nephropathy" with severe hypokalemia caused by renal potassium loss.

There is a general similarity between the patterns of serum lysozyme concentrations in relation to diagnoses reported by several authors using bacteriolytic determination methods (Jollès & al., 1965; Perillie & al., 1968; Wiernik & Serpick, 1969; Pruzanski & Saito, 1969; Youman & al., 1970; Zucker & al., 1970; Senn & Rhomberg, 1970; Asamer & al., 1971a; Catovsky & al., 1971). Leukemias diagnosed as lymphoblastic and chronic lymphocytic have shown normal or subnormal serum lysozyme. There is general agreement that monocytic leukemias tend to display the highest lysozyme concentrations. Authors separating myelomonocytic leukemia from "pure" monocytic leukemia have, in general, found the highest lysozyme values in the latter group. For cases diagnosed as myeloblastic, reported results vary considerably as to the range of serum lysozyme values and the degree of overlap with the monocytic leukemias. These differences are likely to be due to the uncertainties involved in the cytological classification of acute leukemias. As remarked by Cohn (1968), it is difficult to identify a monocyte precursor population in the normal bone marrow, and in leukemias there is a corresponding difficulty in the differentiation of "monoblasts" and monocytes from myeloblasts and promyelocytes (Jollès & al., 1965; Osserman & Lawlor, 1966). In cases with intermediate or mixed cytological features many hematologists apply the designation "myelomonocytic leukemia" (e.g., Saarni & Linman, 1971).

In the present study the diagnosis of monocytic leukemia was made on blood and bone marrow smears without knowledge of lysozyme values or other data. The 7 cases thus selected all had an acute or subacute course. Serum lysozyme values in this group were, on the average, clearly higher than those obtained in the remaining cases of acute leukemia, which were not cytologically subdivided. However, as noted by some other authors, a considerable overlap was evident (Fig. 1 A). In fact, in several of the cases classified as "non-monocytic" acute leukemia occasional cells with somewhat monocytoïd features could be found. A feature of some of the cases classified as monocytic on the basis of peripheral blood morphology was the scarcity in the bone marrow of cells of a similar monocytoïd appearance, and also of cells that could be assumed to be their precursors. Rather, the marrow showed a predominance of promyelocytes. This morphological discrepancy has also been noted by Wintrobe (1967b) and by Meister (1970).

The polycythemia vera patients had elevated serum lysozyme, although in several cases only slightly. For comparison, patients with elevated erythrocyte counts of other etiology were studied; all these had normal serum lysozyme. Binder & Gilbert (1970), who studied a larger number of polycythemia vera patients, found several with serum lysozyme within the normal range.

Renal function must be taken into account in the interpretation of serum and urinary lysozyme values. The small molecular size of lysozyme permits glomerular filtration. Renal handling of lysozyme is like that of other "tubular proteins", i.e. there is almost complete proximal tubular reabsorption followed by proteolytic destruction in the tubular cells (reviewed by Osserman, 1970). Thus it is believed that filtered lysozyme is not to any extent returned to the blood. Proximal tubular injury causes lysozymuria. The utility of urinary lysozyme assays in nephrology will not be discussed here. For the use of lysozyme in hematology it is sufficient to note that tubular dysfunction does not influence serum lysozyme levels, since the amount of lysozyme that has been filtered will in any case be lost from plasma, whether by tubular catabolism or by being excreted in the urine. It is of interest, however, that lysozyme itself seems to be able to produce tubular injury when the filtered load is large due to elevated plasma concentration (Muggia & al., 1969; Pruzanski & Platts, 1970). Thus, a lysozymuria that is basically "overflow" (i.e., caused by a plasma lysozyme level sufficiently raised to make the filtered load exceed tubular reabsorptive capacity) may become compounded by an element of "tubular" lysozymuria; by this mechanism urinary lysozyme excretion will tend to increase in patients with a constant elevation of serum lysozyme. Finally, some patients exhibit lysozymuria although the serum lysozyme concentration is only slightly elevated; in such cases it may be impossible to exclude the presence of preexisting minor renal tubular disease. For this reason it is difficult to define a renal "threshold" for lysozyme. Decreased glomerular filtration, on the other hand, is always of significance as it will cause an elevation of serum lysozyme, causing difficulties in the interpretation of lysozyme values in patients with a concomitant blood disease. Because literature data on serum lysozyme concentrations in relation to glomerular filtration rates are rather fragmentary, in the present studies serum lysozyme determinations have been performed in 46 patients without primary hematological disease,

with various degrees of azotemia. The serum lysozyme - serum creatinine relationship obtained is shown in Fig. 1, paper III.

Apart from their possible differential diagnostic value, serum lysozyme determinations have been examined with regard to usefulness in making prognosis and in following effects of therapy in the acute leukemias. Whereas Wiernik & Serpick (1969) found repeated serum lysozyme determinations to be a valuable adjunct to blood and bone marrow examinations, other authors have reported that variations in lysozyme values are not closely related to the clinical course (Youman & al., 1970; Zucker & al., 1970; Catovsky & al., 1971). In the present studies, the limited serial observations on serum lysozyme have not been included in the report (III). Experience to date would seem to indicate that the clinical value of lysozyme determinations in this regard is rather limited.

LACTOFERRIN IN BLOOD DISORDERS (IV)

The purification of human lactoferrin and initial electrophoretic and immunodiffusion studies with this protein (II) have been referred to previously (pp. 16f.). Lactoferrin could be measured by the radioactive single radial immunodiffusion method described by Rowe (1969). However, the sensitivity of this method in the present system was low, the detection limit being about one mg/l. A further disadvantage of the method is the length of time, about 12 days, needed for the completion of the assay. Of 94 normal sera studied, 24 had an immeasurably low lactoferrin concentration. The upper limit of the normal range was found to be 3.5 mg/l.

In the 50 pathological sera studied, elevated lactoferrin concentrations were found in samples from patients with chronic myelocytic leukemia and polycythemia vera, although only in about half of the cases in each group (Fig. 1 B). In addition, two cases of monocytic leukaemia and one atypical case of chronic lymphocytic leukemia exhibited increased serum lactoferrin. No relations between lactoferrin data and any clinical features were apparent.

In the rabbit heterophil granulocyte, the counterpart of the human neutrophil, lactoferrin has been found to be localized in the

secondary ("specific") granules (Baggiolini & al., 1970b). Although similar data are not available for the human neutrophil, immunofluorescence observations on human bone marrow (Masson & al., 1969) suggest that the maximum cellular content of lactoferrin is not reached until the metamyelocyte stage. This observation is compatible with a localization in secondary rather than primary granules. Thus, myeloblasts and promyelocytes can be expected to contain little or none of this protein. Accordingly, it is reasonable to expect that serum concentrations of lactoferrin, if at all related to leukocyte turnover, should reflect processes involving rather mature cells of the neutrophil series. The observations made, i.e., elevated lactoferrin in chronic myelocytic leukemia and polycythemia vera and normal values in acute leukemia, are in accord with this assumption.

MYELOPEROXIDASE IN BLOOD DISORDERS (V)

Whereas lysozyme and lactoferrin were conveniently available in urine and milk, respectively, myeloperoxidase had to be obtained from leukocytes. In the course of studies by Olsson (1971) on the contents of human granulocyte granules, myeloperoxidase-rich fractions were produced. Such preparations, after purification by gel filtration, were used in the present studies as antigen for the production of rabbit antisera, and as standard for immunochemical determination of this protein.

The assay method used was the radio-immunodiffusion method described by Rowe (1969), i.e., the same as used for the determination of lactoferrin (IV). The immunoprecipitate zones initially obtained with myeloperoxidase were less distinctly outlined than those with lactoferrin, but the inclusion of polyethylene glycol in the immunodiffusion gel resulted in clearly defined radioautographic zones. The lowest serum myeloperoxidase concentration that could be read was 2 mg/l. In none of the 38 normal sera studied did the concentration exceed this limit. The pattern of myeloperoxidase concentrations in sera from 50 patients with leukemia and polycythemia is shown in Fig. 1 C. Nine of the 12 acute (non-monocytic) leukemia

cases had elevated serum myeloperoxidase, in two sera exceeding 10 mg/l. All cases of monocytic leukemia, and the majority of cases of chronic myelocytic leukemia, likewise had elevated values. Five of the 13 polycythemia vera cases showed slight to moderate elevation, whereas in chronic lymphocytic leukemia normal serum myeloperoxidase was observed in all 10 cases.

As shown in Table I, paper V, there was no apparent relation between peripheral leukocyte counts and serum myeloperoxidase concentrations in the acute leukemias. This might be expected, since the peripheral leukocyte picture only poorly reflects this disease process; the majority of the patients had subnormal total leukocyte counts at the time of blood sampling.

Current knowledge on the cellular distribution of myeloperoxidase has been reviewed in paper V. Myeloperoxidase differs from lysozyme and lactoferrin in being restricted to leukocytes. It is present in monocytes and in the neutrophilic granulocyte series.

In the latter, it is localized in the primary cytoplasmic granules, i.e., the granule class appearing at the early promyelocyte stage (Dunn & al., 1968, and others). This is in accordance with the early appearance of cytochemical peroxidase activity in normal granulocytopoiesis. Activity is often seen at the myeloblast stage, i.e., before the appearance of cytoplasmic granulation visible in Romanowsky stains. Leukemic blast cells classed as myeloblasts often display more intense peroxidase activity than their normal counterparts (Hayhoe & al., 1964).

Although strict evidence is lacking, it may provisionally be assumed that an elevated serum myeloperoxidase concentration reflects an increased total number, and/or increased turnover, of monocytes or of any cell type in the maturation line from myeloblasts to neutrophilic granulocytes. If so, the acute leukemias with elevated myeloperoxidase may be presumed to be myeloblastic. It is of interest to note that serum lactoferrin, in contrast to myeloperoxidase, was normal in all acute leukemia cases studied. This finding is in line with the previously discussed data on the appearance of these proteins in the granulocytic maturation chain, and thus lends some support to the assumption that changes in serum levels of these proteins are in fact related to the leukemic process.

DETERMINATION OF LEUKOCYTIC PROTEINS IN LEUKOCYTE EXTRACTS.

Blood leukocytes were isolated by a dextran sedimentation method, as follows:

Forty ml of blood, anticoagulated with heparin, were mixed with 2 ml of a 12% (w/v) solution of dextran (average molecular weight 250 000; Pharmacia, Uppsala, Sweden). After sedimentation for one hour at 4°C the plasma layer was aspirated and centrifuged for 8 min at 400 g in the cold. The cell button was resuspended in 20 ml 0.15 M ammonium chloride, buffered to pH 7.4 with 0.01 M Tris-HCl, and the suspension was left at room temperature for 15 min to achieve lysis of remaining red cells (Dioguardi & al., 1963). After centrifugation for 10 min at 100 g the cell button was resuspended in 20 ml of 1% (w/v) bovine serum albumin (BSA; Sigma Chemical Co., St. Louis, Mo., USA) in 0.15 M NaCl. After a final centrifugation for 5 min at 100 g the cells were taken up in 5 ml BSA. A total white cell count was made in a Bürker chamber and a differential count from a Pappenheim-stained smear.

The cell suspensions were extracted for protein assays as follows: a one-ml aliquot of the suspension was centrifuged and the supernatant carefully drained off, leaving the button of packed cells. One ml of 0.05 M sodium acetate buffer pH 3.7 was added and the cells resuspended. The suspension was left over night at 4°C. It was then subjected to six-fold freezethawing, employing a dry ice-acetone mixture, and finally centrifuged for one hour at 12 000 g. The clear supernatant (extract I) was used, in suitable dilutions, for the determinations of lysozyme, lactoferrin, and myeloperoxidase, employing the same immunochemical methods as with serum samples. Another aliquot of the leukocyte suspension was treated in an identical fashion, except for the inclusion of 1 M NaCl in the acetate buffer (extract II).

Determinations were made with leukocytes from 7 normal individuals and with cells from one case of monocytic leukemia (blood leukocyte count 33 000/ μ l; less than one per cent normal leukocytes). Results are shown in the accompanying table. With normal cells, differences between extract I and extract II were small, extract II giving somewhat higher values; only these latter values are shown. With the leukemic monocytes, extract II contained about twice as much lysozyme as

extract I; both values are shown. For normal leukocytes results have been expressed as micrograms of protein per 10^8 total cells, as well as per 10^8 neutrophilic granulocytes. The latter values were calculated from the differential cell counts of the leukocyte preparations; these differential counts did not differ materially from those of the whole blood samples used for leukocyte preparation. Monocytes have not been included together with the neutrophils, but because of the small percentages of monocytes in the preparations such a calculation would give data only minimally different from those related to neutrophils alone.

LEUKOCYTE CONTENTS OF LYSOZYME, LACTOFERRIN, AND
MYELOPEROXIDASE

Normal individuals and one case of monocytic leukemia

		Amount of protein	
		$\mu\text{g per } 10^8$	$\mu\text{g per } 10^8$
		leukocytes	neutrophils
<u>Normal</u>	LZ	230	430
(average of 7	LF	50	90
individuals)	MPO	60	115
<u>Monocytic</u>	LZ	550-	
<u>leukemia</u>		1150	
	LF	0 ¹	
	MPO	90	

¹less than $3 \mu\text{g}/10^8$ cells

LZ = lysozyme LF = lactoferrin MPO = myeloperoxidase

Thus, leukemic monocytes seem to contain more lysozyme than normal neutrophils. This finding, as well as the data on the lysozyme content of normal leukocytes, are in accordance with results presented by others (Noble & Fudenberg, 1967; Perillie & al., 1968; Senn & al., 1970; Asamer & al., 1971a). In leukemias a rough parallelism has been noted between leukocyte lysozyme content and serum lysozyme level; it is of interest that variations in cell lysozyme seem to involve morphologically normal cells as well as obviously leukemic cells (Asamer & al., 1971a).

The leukocyte lactoferrin content obtained here is only about one-third of that reported by Masson & al. (1969), and for myeloperoxidase the present figures amount to only about one-tenth of those reported by Schultz & Kaminker (1962). There is reason to believe that the present determinations may be underestimates. Whole cell lysates inevitably contain large amounts of nucleic acid. The tendency of lactoferrin to attach to such molecules have been referred to previously, and the behavior of myeloperoxidase in agarose gel makes it probable that this protein also interacts with polyanionic compounds. Thus, the nucleic acid in the extracts may well have interfered with the immunological assay employed.

GENERAL DISCUSSION

As discussed in the Introduction, elevated serum levels of proteins of leukocytic origin can be presumed to reflect the presence of increased numbers of certain leukocytes in the organism (if protein release takes place from viable cells) and/or an increased rate of turnover of such cells (if protein release takes place at the ultimate cell destruction). In the latter case, information is obtained only for that proportion of cells that is destroyed "internally", and perhaps preferentially for cells destroyed at certain anatomic sites.

An additional possibility, not to be entirely dismissed, is that an increased serum concentration of a leukocytic protein may be caused solely by the presence of cells carrying larger than normal amounts of this protein, without any increase in either total cell mass or turnover rate. Such a situation, however, can hardly be envisaged in manifest leukemic disease.

Whereas some release of cellular constituents at cell disintegration can be taken for granted, the additional possibility of leakage from viable cells can be neither confirmed nor refuted, at present. Evidence that the latter process takes place with B_{12} -binding proteins has been referred to previously. In granulocytes, the "degranulation" observed during phagocytosis (Hirsch & Cohn, 1960) involves a fusion of granules with phagocytic vacuoles and consequently a discharge of granule contents into the vacuole; extrusion of granules or their contents to the exterior is not morphologically evident. Nevertheless, some discharge of granule contents from cells has been observed; possible mechanisms have been reviewed by Weissmann & al. (1971). Lysozyme release from leukocytes during phagocytosis or on exposure to bacterial endotoxin has been observed in vitro and in vivo (Kerby & Barrett, 1954; Ribble & Bennett, 1959; Crowder & al., 1969; Tew & al., 1971). Although cell death and disintegration may have occurred to some extent in these experiments, Crowder & al. (1969) and Tew & al. (1971) provided evidence that lysozyme release could take place from viable cells.

An obvious possibility is that leukemic leukocytes may have a disturbance in the granule formation process. In normal cells, synthesis of granule constituents is believed to proceed in close temporal conjunction with the formation of granules to enclose them

(Wetzel & al., 1967; Olsson, 1968). In leukemic leukocytes granule formation may be quantitatively or qualitatively deficient. Particularly in cells containing increased amounts of lysozyme, the synthesis of this protein may outstrip granule formation. The electron microscopic immunocytochemical studies of Sternberger & al. (1970) do, in fact, suggest that lysozyme may be partially extragranular in leukemic cells. If so, leakage to the exterior might be more prone to occur.

The above considerations are of interest for the following reasons. If, in monocytic leukemia with massive lysozymuria, it is assumed that excretion in the urine is the only significant disposal mechanism for lysozyme, knowledge of the amount of lysozyme excreted daily, and of the lysozyme content of the leukemic monocytes, could be used for a calculation of the number of cells destroyed daily ("internally"), an information not easily obtained by other means. However, such a calculation requires proof that lysozyme is discharged only at cell destruction, and that the entire cellular content of lysozyme is delivered to blood plasma, to be transported to the kidneys.

The apparent overproduction of lysozyme in certain leukemic cells is, in itself, of considerable interest. It may be contrasted with the situation in myelomatosis, where the amount of monoclonal immunoglobulin produced is believed to correspond to the size of the neoplastic plasma cell clone, i.e., with no overproduction of immunoglobulin in each cell (Potter, 1967). If leukemic monocytes continuously leak lysozyme the extent of increased lysozyme production is, of course, even greater than is apparent from the amounts of lysozyme present in the cells. Considering that myeloperoxidase is not susceptible to loss from blood plasma through glomerular filtration, the serum concentrations of this protein in monocytic leukemia with very high serum lysozyme (V) are low. Osserman & Lawlor (1966) similarly observed the absence of increased urinary excretion of ribonuclease and other leukocyte constituents in patients with massive lysozymuria, and suggested that the seemingly exclusive capacity for increased lysozyme production may be a consequence of the neoplastic nature of these cells.

The association between myelomatosis and monocytic leukemia deserves comment. One of the cases of monocytic and monomyelocytic leukemia with lysozymuria reported by Osserman & Lawlor (1966) had presented as myelomatosis, and had received treatment with phenylalanine mustard (melphalan). Four years after diagnosis, when the excretion

of Bence Jones protein had decreased significantly, a leukemic blood picture developed and lysozymuria was found. Subsequently, a large number of patients under melphalan treatment for myelomatosis have been observed to develop acute leukemia, most of these with a monocytoïd morphology and associated with raised serum lysozyme with or without lysozymuria (Holland, 1970; Lancet, 1971; Brit. Med. J., 1971). One such case has been observed at this Department (Hällén & Markovich, to be published). Conversely, patients with monocytic leukemia often display increased serum immunoglobulin levels, with or without monoclonal peaks (Osserman & Lawlor, 1966; Pruzanski & Platts, 1970; Malmquist, unpublished). As suggested by Osserman (1971), the explanation may reside in the functional interrelationship between monocytes and plasma cells in the processing of antigen and the formation of antibody. Ordinarily, patients with myelomatosis have normal serum lysozyme (Malmquist, unpublished). An experimental counterpart of these observations is the neoplastic growth induced in NCBxBALB/c mice by intraperitoneal paraffin injections. Whereas the majority of these mice develop immunoglobulin-producing plasmacytomas, in one instance a lysozyme-producing "monocytoma" was obtained (Riblet & Herzenberg, 1970).

Serum levels of leukocytic proteins, in addition to their possible usefulness as indicators of increased leukocyte turnover, are related to leukemic cell type and level of maturation. In the above summaries of the studies on serum leukocytic proteins in leukaemia (III, IV, V) reference has been made to current knowledge on the production of granulocyte granules and their contents. Some differences in opinion on the identification and characteristics of the various granule types exist even for the most extensively studied species, the rabbit (Zeya & Spitznagel, 1971). However, it is generally agreed that there is a temporal succession of production processes during cell maturation. Primary granules containing, among others, myeloperoxidase appear at the promyelocyte stage, whereas secondary granules with alkaline phosphatase as marker are added at the myelocyte stage. One or two additional granule classes have been identified (Spicer & Hardin, 1969; Baggiolini & al., 1970a). For the present discussion the following points are considered to be established for normal leukocytes:

Lysozyme is demonstrable in granulocytopoiesis from the myelocyte stage (e.g., Asamer & al., 1969). In the rabbit it seems to be present

in primary as well as secondary granules (Baggiolini & al., 1970a). Similar data are not available for human granulocytes. Lysozyme is present in normal monocytes as well.

Lactoferrin has been found to be restricted to secondary granules in the rabbit heterophil (Baggiolini & al., 1970b). Observations on human bone marrow are compatible with the same localization in this species (Mason & al., 1969). This protein is not demonstrable in monocytes (Mason & al., 1969).

Myeloperoxidase is a primary granule constituent in rabbit heterophils as well as in human neutrophils (Spicer & Hardin, 1969). A peroxidase with properties indistinguishable from those of the neutrophil enzyme is present in monocytes.

None of these proteins have been found in lymphocytes, platelets, or erythrocytes, or their precursors.

Differences between various cell lines with regard to cytoplasmic constituents are the basis for the cytochemical stains of blood and bone marrow smears widely used as aids in the cytological differential diagnosis of leukemias (Hayhoe, 1969; Hayhoe & al., 1964; Schmalzl & Braunsteiner, 1971). Peroxidase stains (which detect myeloperoxidase in neutrophils and monocytes, as well as eosinophil peroxidase) are employed to identify blast cells as myeloblasts, since lymphoblasts do not contain this enzyme. The limitations of cytochemical stains as means for cell line identification will not be discussed here.

It is obvious that observations on leukocytic proteins in serum may be given interpretations analogous to those of cytochemical stains. Thus, in acute leukemias with elevated serum myeloperoxidase the proliferating cell line may tentatively be identified as myeloblastic, or "monoblastic". The value of serum lysozyme data for the diagnosis of monocytic leukemia is, at present, a matter of discussion. A firm decision on this point requires a reliable independent method for identifying cells as monocytes or "monoblasts". In the opinion of some workers non-specific esterase stains constitute such a method (Schmalzl & Braunsteiner, 1971). However, the combined use of cytochemical methods, serum lysozyme determinations, and estimation of cellular lysozyme content by immunofluorescence did not effect a clear separation of monocytic from promyelocytic leukemias (Asamer & al., 1971b). If, as suggested (Leder, 1967), monocytes actually originate in the granulocyte maturation chain, a strict distinction of monocytic leukemias from immature myeloid leukemias may not be possible or meaningful. In the present work (III) the separation, in Pappenheim-

stained smears, of atypical or immature monocytes from other blast forms was often found to be difficult and to some extent arbitrary. However, there is no doubt that, regardless of specific cell line identification, the highest serum lysozyme levels are seen in leukemias where monocyte-like cells are in obvious predominance.

In addition to the possibility of cell line identification, specialized cellular constituents may to some extent be informative of degree of maturation, as various components may appear at different maturation stages. Literature data for granulocyte maturation have been referred to previously. As was likewise pointed out (p. 24), the patterns of serum lactoferrin and myeloperoxidase concentrations in relation to diagnoses are in accord with the maturation level (acute leukemia vs. chronic myelocytic leukemia).

Further work will be needed to establish whether estimations of leukocytic proteins in body fluids are valuable for the identification of leukemic "cell species", or for assessing leukemic cell pool sizes and turnover rates.

SUMMARY

The present studies comprise the following:

- I. The isolation of human urinary lysozyme and the elaboration of an immunochemical method for the measurement of lysozyme in serum and urine. The normal serum lysozyme range was found to be 1 - 5 mg/l.
- II. Physico-chemical studies with lactoferrin purified from human milk. The protein was found to interact markedly with agar gels and with some dye compounds.
- III. Studies on serum and urinary lysozyme in hematological disease, and on serum lysozyme in azotemia. The importance of reduced glomerular filtration as a cause of small to moderate elevations in serum lysozyme was emphasized. Serum and urinary lysozyme concentrations in relation to hematological diagnoses were found to be largely similar to those obtained by other authors using bacteriolytic lysozyme determination. The highest serum lysozyme concentrations were found in cases of monocytic leukemia, but considerable overlap between this group and other acute leukemias was evident. The difficulties involved in the recognition of "atypical monocytes", as opposed to other blast cells, were pointed out.
- IV. The measurement of lactoferrin in serum by a radio-immunodiffusion method. The detection limit of the method was 1 mg/l. Normal sera contained up to 3.5 mg/l of this protein. Elevated concentrations were largely restricted to sera from patients with chronic myelocytic leukemia and polycythemia vera.
- V. The measurement of myeloperoxidase in serum by a similar radio-immunodiffusion method. Myeloperoxidase was obtained from human myelocytes and granulocytes. The lower working limit of the immunoassay was 2 mg/l. In normal sera concentrations exceeding this limit were not found. Elevated serum concentrations were found in the majority of cases of acute leukemia, in all cases of monocytic leukemia, and in a minority of cases of chronic myelocytic leukemia and polycythemia vera.

The results have been evaluated in relation to present knowledge on the appearance of these proteins in the granulocyte maturation sequence. With regard to cytological differential diagnosis in leukemias, and the assessment of leukemic cell masses and turnover rates, the findings have been discussed as possible supplements to other clinical methods.

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REFERENCES

- Agner, K.: Verdoperoxidase. A ferment isolated from leucocytes. *Acta physiol. scand.* 2: Suppl. 8, 1941.
- Alderton, G., Ward, W.H. & Fevold, H.L.: Isolation of lysozyme from egg white. *J. biol. Chem.* 157: 43, 1945.
- Ambrus, C.M. & Ambrus, J.L.: Regulation of the leukocyte level. *Ann. N.Y. Acad. Sci.* 77: 445, 1959.
- Asamer, H., Schmalzl, F. & Braunsteiner, H.: Der immunzytologische Lysozymnachweis in menschlichen Blutzellen. *Acta haemat. (Basel)* 41: 49, 1969.
- Asamer, H., Schmalzl, F. & Braunsteiner, H.: Die diagnostische und prognostische Bedeutung der Muramidase-(Lysozym-)Bestimmung in Leukocytenlysaten, Serum und Harn von Leukämiepatienten. *Klin. Wschr.* 49: 587, 1971a.
- Asamer, H., Schmalzl, F. & Braunsteiner, H.: Immunocytological demonstration of lysozyme (muramidase) in human leukaemic cells. *Brit. J. Haemat.* 20: 571, 1971b.
- Baggiolini, M., Hirsch, J.G. & de Duve, C.: Resolution of granules from rabbit heterophil leukocytes into distinct populations by zonal sedimentation. *J. Cell Biol.* 40: 529, 1969.
- Baggiolini, M., Hirsch, J.G. & de Duve, C.: Further biochemical and morphological studies of granule fractions from rabbit heterophil leukocytes. *J. Cell Biol.* 45: 586, 1970a.
- Baggiolini, M., de Duve, C., Masson, P.L. & Heremans, J.F.: Association of lactoferrin with specific granules in rabbit heterophil leukocytes. *J. exp. Med.* 131: 559, 1970b.
- Begemann, H.: *Klinische Hämatologie*, p. 601. Georg Thieme, Stuttgart, 1970.
- Beeson, P.B.: Effect of reticulo-endothelial blockade on immunity to the Shwartzman phenomenon. *Proc. Soc. Exp. Biol. (N.Y.)* 64: 146, 1947.
- Binder, R.A. & Gilbert, H.S.: Muramidase in polycythemia vera. *Blood* 36: 228, 1970.
- Boggs, D.R.: The kinetics of neutrophilic leukocytes in health and disease. *Semin. Hemat.* 4: 359, 1967.
- Boggs, D.R., Athens, J.W., Haab, O.P., Raab, S.O., Cartwright, G.E. & Wintrobe, M.M.: Leukokinetic studies. VIII. A search for an extramedullary tissue pool of neutrophilic granulocytes. *Proc. Soc. Exp. Biol. (N.Y.)* 115: 792, 1964.

- Briggs, R.S., Perillie, P.E. & Finch, S.C.: Lysozyme in bone marrow and peripheral blood cells. *J. Histochem. Cytochem.* 14: 167, 1966.
- Brit. Med. J. (editorial): Leukaemia on myeloma. *Brit. Med. J.* 1: 568, 1971.
- Cartwright, G.E., Athens, J.W. & Wintrobe, M.M.: The kinetics of granulopoiesis in normal man. *Blood* 24: 780, 1964.
- Catovsky, D., Galton, D.A.G. & Griffin, C.: The significance of lysozyme estimations in acute myeloid and chronic monocytic leukaemia. *Brit. J. Haemat.* 21: 565, 1971.
- Chikkappa, G., Corcino, J., Greenberg, M.L. & Herbert, V.: Correlation between various blood white cell pools and the serum B₁₂-binding capacities. *Blood* 37: 142, 1971.
- Clarkson, B.D.: Review of recent studies of cellular proliferation in acute leukemia. *In* Perry, S. (Ed.): *Human Tumor Cell Kinetics*. National Cancer Institute Monograph 30, p. 81. U.S. Govt. Printing Office, Washington, D.C., 1969.
- Cohn, Z.A.: The structure and function of monocytes and macrophages. *Adv. Immunol.* 9: 163, 1968.
- Craddock, C.G.: The physiology of granulocytic cells in normal and leukemic states. *Amer. J. Med.* 28: 711, 1960.
- Crowder, J.G., Martin, R.R. & White, A.: Release of histamine and lysosomal enzymes by human leukocytes during phagocytosis of staphylococci. *J. Lab. clin. Med.* 74: 436, 1969.
- Dioguardi, N., Agostini, A., Fiorelli, G. & Lomanto, B.: Characterization of lactic dehydrogenase of normal human granulocytes. *J. Lab. clin. Med.* 61: 713, 1963.
- Dumont, A.E. & Weissmann, G.: Lymphatic transport of beta-glucuronidase during haemorrhagic shock. *Nature (London)* 201: 1231, 1964.
- Dunn, W.B., Hardin, J.H. & Spicer, S.S.: Ultrastructural localization of myeloperoxidase in human neutrophil and rabbit heterophil and eosinophil leukocytes. *Blood* 32: 935, 1968.
- Eisenstein, R. & Kuettner, K.C.: An ultrastructural organization of cartilage matrix as revealed through the use of lysozyme. *Calc. Tissue Res.* 4 (Suppl.): 137, 1970.
- Finch, S.C., Ghabasik, F.J. & Rogaway, W.: Lysozyme and leukopoiesis. *Proc. 3rd Intern. Symp. Fleming's Lysozyme*, p. 137. Milan, 1964.
- Fliedner, T.M., Cronkite, E.P. & Robertson, J.S.: Granulocytopoiesis. I. Senescence and random loss of neutrophilic granulocytes in human beings. *Blood* 24: 402, 1964.

- Flidner, T.M., Laeger, F. & Cronkite, E.P.: Zytokinetische Untersuchungen an menschlichen Blutmonozyten. In Brücher, H. (Ed.): Der Monozyt, p. 39. J.F. Lehmanns Verlag, München, 1969.
- Galbraith, P.R., Chikkappa, G. & Abu-Zahra, H.T.: Patterns of granulocyte kinetics in acute myelogenous and myelomonocytic leukemia. Blood 36: 371, 1970.
- Gillman, J. & Gillman, T.: Lymphomata (including Hodgkin's-like sarcomata). Their experimental production. Clin. Proc. (Cape Town) 8: 222, 1949.
- Gorin, G., Wang, S.-F. & Papapavlou, L.: Assay of lysozyme by its lytic activity on M. lysodeikticus cells. Analyt. Biochem. 39: 113, 1971.
- Harrison, J.F. & Swingler, M.: The effects of serum macromolecules on the lysis of Micrococcus lysodeikticus cells by lysozyme. Clin. Chim. Acta 31: 149, 1971.
- Hayhoe, F.G.J.: The cytochemistry of haemopoiesis. In Britton, C.J.C.: Disorders of the Blood, 10th Ed., p. 123. J.&A. Churchill, Ltd, London, 1969.
- Hayhoe, F.G.J., Quaglino, D. & Doll, R.: The Cytology and Cytochemistry of Acute Leukaemias. Medical Research Council Special Report Series No. 304, p. 86-87. Her Majesty's Stationery Office, London, 1964.
- Hirsch, J.G.: Neutrophil and eosinophil leucocytes. In Zweifach, B.W., Grant, L. & McCluskey, R.T. (Eds.): The Inflammatory Process, p. 245. Academic Press, New York & London, 1965.
- Hirsch, J.G. & Cohn, Z.A.: Degranulation of polymorphonuclear leukocytes following phagocytosis of microorganisms. J. exp. Med. 112: 1005, 1960.
- Holland, J.F. (editorial): Epidemic acute leukemia. New Engl. J. Med. 283: 1165, 1970.
- Inai, S., Hirao, F., Kishimoto, S. & Takahashi, H.: Studies on serum lysozyme II. Lysozyme activity in normal human serum and in serum of patients with various diseases. Med. J. Osaka Univ. 9: 33, 1958.
- Johansson, B.G.: Isolation of an iron-containing red protein from human milk. Acta chem. scand. 14: 510, 1960.
- Johansson, B.G.: Isolation of crystalline lactoferrin from human milk. Acta chem. scand. 23: 683, 1969.
- Jollès, J. & Jollès, P.: La structure chimique primaire du lysozyme du lait de femme: établissement d'une formule développée provisoire. Helv. Chim. Acta. 52: 2671, 1969.

- Jollès, P.: Lysozymes: a chapter of molecular biology. *Angew. Chem. Internat. Edit.* 8: 227, 1969.
- Jollès, P., Sternberg, M. & Mathé, G.: Study of the lysozymic content of the serum in patients with leukaemias and haematosarcomas. *Proc. 3rd Intern. Symp. Fleming's Lysozyme*, p. 138. Milan, 1964.
- Jollès, P., Sternberg, M. & Mathé, G.: The relationship between serum lysozyme levels and the blood leukocytes. *Israel J. Med. Sci.* 1: 445, 1965.
- Kerby, G.P. & Barrett, J.A.: The effect of hydrocortisone and of Piromen in vitro on leukocytes of patients receiving ACTH and cortisone therapy. *J. clin. Invest.* 33: 725, 1954.
- Klebanoff, S.J.: Myeloperoxidase: contribution to the microbicidal activity of intact leukocytes. *Science* 169: 1095, 1970.
- Krakoff, I.H. & Baliss, M.E.: Abnormalities of purine metabolism in human leukemia. *Ann. N.Y. Acad. Sci.* 113: 1043, 1964.
- Lancet (editorial): Leukaemia and cytotoxic drugs. *Lancet* I: 70, 1971.
- Laurell, C.-B.: Quantitative estimation of proteins by electrophoresis in agarose gel containing antibodies. *Analyt. Biochem.* 15: 45, 1966.
- Leder, L.-D.: The origin of blood monocytes and macrophages. *Blut* 16: 86, 1967.
- Lloyd, J.B. & Beck, F.: Teratogenesis. *In* Dingle, J.T. & Fell, H.B. (Eds.): *Lysosomes in Biology and Pathology*, Vol. 1, p. 433. North-Holland Publ. Co., Amsterdam & London, 1969.
- Loisillier, F., Burtin, P. & Grabar, P.: Isolement et caractérisation de l'auto-antigène responsable de la formation d'auto-anticorps chez les malades atteints de lésions mammaires (cancéreuses ou non). *Ann. Inst. Pasteur* 115: 829, 1968.
- Magill, G.B., Wroblewski, F. & LaDue, J.S.: Serum lactic dehydrogenase and serum transaminase in human leukemia. *Blood* 14: 870, 1959.
- Maron, E. & Bonavida, B.: A sensitive immunoassay for human lysozyme in biological fluids. *Biochim. Biophys. Acta* 229: 273, 1971.
- Masson, P.L.: *La Lactoferrine*. Arscia, Bruxelles, 1970.
- Masson, P.L., Heremans, J.F. & Schonke, E.: Lactoferrin, an iron-binding protein in neutrophilic leukocytes. *J. exp. Med.* 130: 643, 1969.
- Meister, H.: Zur Zytochemie der Monozytenleukose. *Haematologia (Budapest)* 4: 311, 1970.
- Montreuil, J., Tonnelat, J. & Mullet, S.: Préparation et propriétés de la lactosidérophiline (lactotransferrine) du lait de femme. *Biochim. Biophys. Acta* 45: 413, 1960.

- Mouton, A. & Jollès, J.: On the identity of human lysozymes isolated from normal and abnormal tissues or secretions. *FEBS Letters* 4: 337, 1969.
- Muggia, F.M., Heinemann, H.O., Farhangi, M. & Osserman, E.F.: Lysozymuria and renal tubular dysfunction in monocytic and myelomonocytic leukemia. *Amer. J. Med.* 47: 351, 1969.
- Noble, R.E. & Fudenberg, H.H.: Leukocyte lysozyme activity in myelocytic leukemia. *Blood* 30: 465, 1967.
- Olsson, I.: Glycosaminoglycans of human leukocytes. Identification, biosynthesis and intracellular distribution. Lund, 1968.
- Olsson, I., 1971. To be published.
- Osserman, E.F.: Lysozymuria in renal and non-renal diseases. *In* Manuel, Y., Revillard, J.P. & Betuel, H. (Eds.): *Proteins in Normal and Pathological Urine*, p. 260. S. Karger, Basel, 1970.
- Osserman, E.F.: Monocytic and monomyelocytic leukaemia with increased serum and urine lysozyme as a late complication in plasma cell myeloma. *Brit. Med. J.* 2: 327, 1971.
- Osserman, E.F. & Lawlor, D.P.: Serum and urinary lysozyme (muramidase) in monocytic and monomyelocytic leukemia. *J. exp. Med.* 124: 921, 1966.
- Perillie, P.E., Kaplan, S.S., Lefkowitz, E., Rogaway, W. & Finch, S.C.: Studies of muramidase (lysozyme) in leukemia. *J. Amer. med. Ass.* 203: 317, 1968.
- Perry, S.: The formation and destruction of leukocytes. *In* Greenwalt, T.J. & Jamieson, G.A. (Eds.): *Formation and Destruction of Blood Cells*, p. 194. J.B. Lippincott, Philadelphia, 1971.
- Perry, S., Goodwin, H.A. & Zimmerman, T.S.: Physiology of the granulocyte. *J. Amer. med. Ass.* 203: 937, 1025, 1968.
- Pitzurra, M. & Frascarelli, R.: La diapedesi leucocitaria attraverso le mucose: intensità e significato del fenomeno nel normale. *Haematologica* 25: 389, 1943.
- Post, J. & Hoffman, J.: *In vivo* replication of normal and tumor cells: relation to cancer chemotherapy. *In* Perry, S. (Ed.): *Human Tumor Cell Kinetics*. National Cancer Institute Monograph 30, p. 209. U.S. Govt. Printing Office, Washington, D.C., 1969.
- Potter, M.: The plasma cell tumors and myeloma proteins of mice. *In* Busch, H. (Ed.): *Methods in Cancer Research*, Vol. 2, p. 105. Academic Press, New York, 1967.
- Pruzenski, W. & Saito, S.G.: The diagnostic value of lysozyme (muramidase) estimation in biological fluids. *Amer. J. med. Sci.* 258: 405, 1969.

- Pruzanski, W. & Platts, M.E.: Serum and urinary proteins, lysozyme (muramidase), and renal dysfunction in mono- and myelomonocytic leukemia. *J. clin. Invest.* 49: 1694, 1970.
- Querinjean, P., Masson, P.L. & Heremans, J.F.: Molecular weight, single-chain structure and amino acid composition of human lactoferrin. *Eur. J. Biochem.* 20: 420, 1971.
- Ribble, J.C. & Bennett, I.L.: Lysozyme release as an indication of in vivo leukocyte injury by endotoxin. *Clin. Res.* 7: 41, 1959.
- Riblet, R.J. & Herzenberg, L.A.: Mouse lysozyme production by a monocytoma: isolation and comparison with other lysozymes. *Science* 168: 1595, 1970.
- Rowe, D.S.: Radioactive single radial diffusion: a method for increasing the sensitivity of immunochemical quantification of proteins in agar gel. *Bull. Wld Hlth Org.* 40: 613, 1969.
- Rytömaa, T. & Teir, H.: Relationship between tissue eosinophils and peroxidase activity. *Nature (London)* 192: 271, 1961.
- Saarni, M.I. & Linman, J.W.: Myelomonocytic leukemia: disorderly proliferation of all marrow cells. *Cancer* 27: 1221, 1971.
- Salmon, S.E., Cline, M.E., Schultz, J. & Lehrer, R.I.: Myeloperoxidase deficiency. Immunologic study of a genetic leukocyte defect. *New Engl. J. Med.* 282: 250, 1970.
- Sandberg, A.A., Cartwright, G.E. & Wintrobe, M.M.: Studies on leukemia. I. Uric acid excretion. *Blood* 11: 154, 1956.
- Schmalzl, F. & Braunsteiner, H.: The application of cytochemical methods to the study of acute leukemia. A review. *Acta haemat. (Basel)* 45: 209, 1971.
- Schultz, J. & Kaminker, K.: Myeloperoxidase of the leucocyte of normal human blood. I. Content and localization. *Arch. Biochem.* 96: 465, 1962.
- Schultz, J. & Shmukler, H.W.: Myeloperoxidase of the leucocyte of normal human blood. II. Isolation, spectrophotometry, and amino acid analysis. *Biochem. (Wash.)* 3: 1234, 1964.
- Schultz, J., Corlin, R., Oddi, F., Kaminker, K. & Jones, W.: Myeloperoxidase of the leucocyte of normal human blood. III. Isolation of the peroxidase granule. *Arch. Biochem.* 111: 73, 1965.
- Schultz, J. & Berger, S.J.: Myeloperoxidase and the biological properties of the "peroxo-lysosome" of the normal human neutrophil. In Schultz, J. (Ed.): *Biochemistry of the Phagocytic Process*, p. 115. North-Holland Publ. Co., Amsterdam & London, 1970.

- Senn, H.J., Chu, B., O'Malley, J. & Holland, J.F.: Experimental and clinical studies on muramidase (lysozyme) I. Muramidase activity of normal human blood cells and inflammatory exudates. *Acta haemat.* (Basel) 44: 65, 1970.
- Senn, H.J. & Rhomberg, W.U.: Muramidaseaktivität in Serum und Urin bei akuten und chronischen Leukämien. *Schweiz. med. Wschr.* 100: 1993, 1970.
- Smith, J.B., Pedersen, N.C. & Morris, B.: The role of the lymphatic system in inflammatory responses. *Series Haematol.* (Copenhagen) III:2: 17, 1970.
- Spicer, S.S. & Hardin, J.H.: Ultrastructure, cytochemistry, and function of neutrophil leukocyte granules. A review. *Lab. Invest.* 20: 488, 1969.
- Sternberger, L.A., Osseman, E.F. & Seligman, A.M.: Lysozyme and fibrinogen in normal and leukemic blood cells: a quantitative electron immunocytochemical study. *Hopkins Med. J.* 126: 188, 1970.
- Syrén, E. & Raeste, A.-M.: Identification of blood monocytes by demonstration of lysozyme and peroxidase activity. *Acta haemat.* (Basel) 45: 29, 1971.
- Teir, H., Rytömaa, T., Cederberg, A. & Kiviniemi, K.: Studies on the elimination of granulocytes in the intestinal tract in rat. *Acta path. microbiol. scand.* 59: 311, 1963.
- Tew, J.G., Scott, R.L. & Donaldson, D.M.: Plasma β -lysin and lysozyme following endotoxin administration and the generalized Schwartzman phenomenon. *Proc. Soc. Exp. Biol. (N.Y.)* 136: 473, 1971.
- Undritz, E.: Die Alius-Grignaschi-Anomalie: Der erblich-konstitutionelle Peroxydasedefekt der Neutrophilen und Monozyten. *Blut* 14: 129, 1966.
- Volkman, A.: The origin and fate of the monocyte. *Series Haematol.* (Copenhagen) III:2: 62, 1970.
- Weissmann, G., Dukor, P. & Zurier, R.B.: Effect of cyclic AMP on release of lysosomal enzymes from phagocytes. *Nature New Biol.* 231: 131, 1971.
- Wetzel, B.K., Horn, R.G. & Spicer, S.S.: Fine structural studies on the development of heterophil, eosinophil, and basophil granulocytes in rabbits. *Lab. Invest.* 16: 349, 1967.
- Wiernik, P.H. & Serpick, A.A.: Clinical significance of serum and urinary muramidase activity in leukemia and other hematologic malignancies. *Amer. J. Med.* 46: 330, 1969.
- Wintrobe, M.M.: *Clinical Hematology*, 6th Ed., p. 259. Lea & Febiger, Philadelphia, 1967a.

- Wintrobe, M.M.: Clinical Hematology, 6th Ed., p. 1028. Lea & Febiger, Philadelphia, 1967b.
- Yam, L.T., Li, C.Y. & Crosby, W.H.: Cytochemical identification of monocytes and granulocytes. Amer. J. clin. Path. 55: 283, 1971.
- Youman, J.D., Saarni, M.I. & Linman, J.W.: Diagnostic value of muramidase (lysozyme) in acute leukemia and preleukemia. Mayo Clin. Proc. 45: 219, 1970.
- Zeya, H.I. & Spitznagel, J.K.: Isolation of polymorphonuclear leukocyte granules from rabbit bone marrow. Lab. Invest. 24: 237, 1971.
- Zucker, S., Hanes, D.J., Vogler, W.R. & Eanes, R.Z.: Plasma muramidase: a study of methods and clinical applications. J. Lab. clin. Med. 75: 83, 1970.

